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Reproducibility of Laboratory Assays for Steroid Hormones and Sex Hormone-binding Globulin

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ABSTRACT

The relationship of serum hormones to cancer risk has recently been pursued in epidemiological studies, but few have reported on the reproducibility of laboratory findings. Prior to conducting a study of endogenous hormones and endometrial cancer, we evaluated the reproducibility of measurements for several hormones (estrone, estradiol, free estradiol, albumin-bound estradiol, and androstenedione) and sex hormone-binding globulin. We obtained a single unit of blood from each of six women and prepared aliquots of serum for repeated testing. Three laboratories analyzed multiple samples on consecutive working days from which estimates of intraassay and interassay measurement variability were obtained. For estrone and estradiol, a log transformation of the data produced distributions which were nearly normal and permitted the use of parametric statistical tests. In general, we found measurements for most hormones varied considerably between assays. Moreover, differences were observed in the absolute values of sex hormone-binding globulin and of the hormones, particularly for estrone and estradiol, from one laboratory to the next. Our findings suggest that variability of current laboratory procedures may hamper efforts to study the association between disease and endogenous hormones in epidemiological studies. In addition, validation of hormone assays is essential in order to assure standardized results and enable comparisons of data across studies.

INTRODUCTION

Circulating hormone levels have increasingly been the focus of clinical laboratory, and epidemiological studies, particularly for cancers of the breast (reviewed in Refs. 1 and 2), prostate (reviewed in Ref. 3) , and endometrium (4–7). Since imprecision in the estimation of hormone levels may obscure biological differences, efforts need to be made to evaluate the validity and reproducibility of the measurements. Unlike clinical settings, where laboratories need only identify extreme hormone values to diagnose abnormalities, epidemiological studies seek to link disease risk to different hormonal levels that fall within normal ranges. To this end, levels of hormones must be measured precisely and consistently.

Sources of variability may arise from laboratory procedures as well as host factors such as age, race, smoking, diet, or menstrual cycle variation. While experimental design and statistical methods can account for some of these characteristics, few studies have addressed the issue of variability across time or of the laboratory analyses themselves. Two studies (8, 9) that evaluated sex hormone concentrations from repeated samples of postmenopausal women over time concluded that the reproducibility of estrogen measurements within subjects, particularly of total estradiol, was relatively poor. However, because a single sample was obtained at each time point, the contribution of laboratory variability could not be assessed. In another methodological study, laboratory variability was evaluated by repeated testing of the same sample within the same laboratory, and reproducibility was poor for a variety of steroid hormones (10).

Prior to the conduct of a study of endogenous hormones and endometrial cancer, we decided to quantitate reproducibility of assays for several hormones in pre- and postmenopausal women.

MATERIALS AND METHODS

Each of three laboratories measured estrone, E2,[2] free estradiol, albumin-bound estradiol, androstenedione and SHBG in serum from six individuals (Table 1, a–f). To examine a spectrum of estradiol levels, blood was obtained from two individuals each in three different menstrual groupings; mid-follicular, preovulatory, and postmenopausal. Individuals donated a unit of whole blood from which serum was prepared and portioned into 2-ml cryovials and frozen until analysis. Serum was mixed throughout the portioning of aliquots to ensure homogeneity across specimens. We requested that each laboratory analyze six aliquots per day for 10 consecutive working days for each hormone or transport protein assay. Thus, each day, duplicate aliquots from three women were assayed. Laboratories were blinded as to the design and menstrual groupings.

The individuals donating blood samples were normal volunteers. Eligible premenopausal women had regular intervals between menstrual periods (28–32 days), were not pregnant, lactating, or using oral contraceptives. Mid-follicular phase blood samples were obtained on days 5–7 of the menstrual cycle, whereas preovulatory samples were obtained 15 days prior to the next menses. Postmenopausal women had experienced a natural menopause with no menses for at least 1 year, had an intact uterus, and did not use exogenous estrogens. Prior to this study, 6 of 15 women who were initially screened were selected based on estradiol measurements. Samples from women in the mid-follicular phase with estradiol concentrations less than 100 pg/ml were chosen, whereas samples from preovulatory phase women with estradiol concentrations above 200 pg/ml were selected.

Three laboratories with extensive experience in hormone assays participated in this study. Each laboratory extracted the steroids from serum, used celite column chromatography to separate hormones, and used radioimmunoassay techniques to measure estrone, estradiol, and androstenedione levels. Laboratory A used diethyl ether (10 volumes) for the extraction, Lab B used two extractions with cyclohexane:ethyl acetate 2:1, and Lab C used 20% ethyl acetate in hexane. Prior to this study, Labs A, B, and C stated their intraassay CVs were 8, 3, and 9% for estrone; 9, 4, and 9% for estradiol; and 3, 8, and 10% for androstenedione, respectively. The laboratory assays differed for free and bound estradiol and SHBG. Lab A measured SHBG (mol/liter $\times 10^{-8}$) and the percentage of free E_2 using a sepharose equilibrium assay first described by Pearlman *et al.* (11, 12) for the measurement of testosterone binding in serum. Albumin-bound E_2 (pg/ml) was estimated as the difference between total E_2, SHBG-E_2, and free E_2. Intraassay CVs for SHBG, percentage of free E_2, and albumin-bound E_2 were stated as 5, 2, and 9%, respectively. Lab B used a radioimmunometric kit to measure SHBG (nmol) and centrifugal ultrafiltration to determine the percentage of free E_2 and the percentage of albumin-bound E_2 as described by Hammond *et al.* (13). Within-assay CVs were reported to be 2, 5, and 6% for SHBG, the percentage of free E_2, and albumin-bound E_2, respectively. Lab C also used ammonium sulfate precipitation to estimate SHBG (binding capacity for dihydrotestosterone) and albumin-bound E_2. SHBG was reported as "μg dihydrotestosterone bound/dl," and albumin-bound E_2 was reported in pg/ml. The percentage of free E_2 was estimated by an equilibrium dialysis assay that assumes binding constants and albumin concentrations. Intraassay CVs were 11, 2, and 1% for SHBG, the percentage of free E_2, and albumin-bound E_2, respectively. Depending on the assay, labora-

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[2] The abbreviations used are: E_2, estradiol; SHBG, sex hormone-binding globulin; CV, coefficient of variation; ANOVA, analysis of variance.

Table 1 *Design for testing samples from six individuals by each laboratory (Hormone Feasibility Trial)*

Day of assay	Individuals						Total samples
	Follicular		Preovulatory		Postmenopausal		
	a	b	c	d	e	f	
1	aa	bb		dd			6
2			cc		ee	ff	6
3	aa		cc	dd			6
4		bb		dd	ee		6
5	aa		cc			ff	6
6		bb			ee	ff	6
7			cc	dd	ee		6
8	aa	bb				ff	6
9	aa	bb		dd			6
10			cc		ee	ff	6
TOTAL ASSAYS	10	10	10	10	10	10	60

Table 2 *Mean (SD) hormone values for six subjects[a] by laboratory (Hormone Feasibility Trial)*

Hormone	Subject	Lab A	Lab B	Lab C
Estrone (pg/ml)	a	72.6 (13.1)	81.8 (17.1)	74.3 (6.0)
	b	29.2 (5.5)	60.6 (14.7)	33.9 (3.0)
	c	95.1 (15.3)	85.0 (20.5)	89.6 (5.6)
	d	109.4 (13.9)	112.9 (18.8)	102.8 (9.0)
	e	21.8 (8.3)	41.4 (11.4)	23.9 (2.0)
	f	21.7 (7.0)	51.3 (10.0)	26.4 (3.1)
Estradiol (pg/ml)	a	96.0 (20.4)	71.2 (8.7)	82.5 (7.5)
	b	69.6 (15.9)	57.0 (9.7)	58.0 (2.3)
	c	304.8 (48.8)	131.5 (27.2)	237.2 (18.5)
	d	309.8 (71.1)	133.3 (23.4)	251.7 (22.7)
	e	17.7 (8.1)	26.1 (5.9)	4.1 (0.6)
	f	9.2 (5.1)	32.3 (16.2)	4.8 (0.4)
Androstenedione (ng/dl)	a	126.4 (24.4)	138.8 (6.5)	127.9 (11.4)
	b	96.1 (21.6)	123.1 (7.1)	108.7 (5.1)
	c	84.7 (14.2)	115.5 (11.0)	104.2 (8.8)
	d	143.3 (27.2)	143.9 (6.1)	186.9 (15.2)
	e	51.8 (18.6)	102.8 (9.0)	57.8 (5.4)
	f	28.1 (13.3)	54.2 (17.8)	28.9 (3.2)
Free E_2 (percentage)	a	1.29 (0.07)	1.23 (0.16)	2.05 (0.07)
	b	1.34 (0.09)	1.34 (0.15)	2.24 (0.04)
	c	1.24 (0.09)	1.11 (0.10)	1.91 (0.06)
	d	1.36 (0.06)	1.46 (0.14)	2.38 (0.07)
	e	1.20 (0.10)	1.23 (0.11)	1.88 (0.07)
	f	1.12 (0.14)	1.09 (0.17)	1.83 (0.07)
Albumin-bound E_2 (pg/ml)	a	27.6 (6.9)	10.6 (1.6)	16.7 (1.8)
	b	21.8 (6.4)	9.1 (1.6)	13.4 (1.5)
	c	77.1 (15.0)	16.7 (3.8)	38.2 (3.2)
	d	129.6 (36.0)	24.5 (4.7)	92.7 (10.0)
	e	5.7 (2.5)	4.2 (1.1)	1.2 (0.2)
	f	2.2 (1.2)	4.4 (2.0)	0.8 (0.1)
SHBG (nmol)	a	22.1 (2.9)	67.4 (7.2)	68.0 (8.5)
	b	19.9 (3.5)	51.6 (4.9)	62.4 (6.2)
	c	24.7 (4.4)	82.1 (10.1)	72.6 (17.0)
	d	19.4 (2.4)	37.0 (4.1)	44.0 (11.6)
	e	25.9 (4.8)	71.9 (20.6)	71.1 (19.7)
	f	30.4 (7.0)	74.0 (9.9)	73.3 (17.4)

[a] Serum from subjects a and b were obtained during the mid-follicular phase of the menstrual cycle; subjects c and d, from the preovulatory phase; and subjects e and f were postmenopausal.

tories reported interassay CVs that were slightly higher than intraassay CVs and ranged between 4 and 15%. All laboratories routinely repeated assays of duplicate estimates that differed by 15% or more. Standard internal quality control procedures were also followed: *e.g.*, assays were repeated if two of three commercial standards were more than 15% different from each other or if control bloods were more than 2 SDs from the mean. Assay values for each hormone or SHBG were converted into the same units for comparisons across laboratories. Serum cholesterol and triglycerides were measured by one laboratory to assess possible influences of blood lipids on the laboratory hormone assays.

A nested (within-subject) ANOVA was used to test the reproducibility of results and to obtain variance estimates for within and between assays, the so-called intraassay and interassay CVs. We also used a nested ANOVA to address the correlation of the same sample being assayed repeatedly. For each hormone, the intraassay CV was calculated by dividing the square root of the within-day variance by the mean hormone level; the interassay CV was calculated similarly, using the square root of the between-day variance estimate. Because of the limited number of repetitions, the variance estimates from the ANOVA model may have resulted in values for the interassay CVs that were lower than the intraassay CVs. The overall CVs were calculated using the sum of the interassay and intraassay variance estimates, ignoring any between-person variance.

RESULTS

The laboratory results are summarized in Table 2, which presents the mean and SD from 10 measurements for each individual obtained

over 5 days. The absolute values of the hormones varied considerably between laboratories, notably for estrone and estradiol. For example, estradiol values ranged form 132 to 305 pg/ml for subject c. The range of hormone values across individuals also differed between labs; estradiol values varied 5-fold for Labs A and C and only 3-fold for Lab B. Furthermore, the relative difference in some hormone levels between women in the same menstrual phase was markedly different between laboratories. Estrone values for the postmenopausal women (subjects e and f) were nearly identical for Lab A but differed by nearly 25% for Lab B. For some hormones, the mean values were similar from all labs, but the variability differed considerably. Mean estrone concentrations for subject c, for example, were approximately 90 pg/ml in all labs, but the SDs ranged from 5.6 and 20.5.

Values of estradiol in the postmenopausal women (subjects e and f) varied amongst labs, with the highest levels reported by Lab B (values of 26.1 and 32.3 pg/ml) and the lowest values by Lab C (4.1 and 4.8 pg/ml). The ratio of E_2 to estrone, which is expected to range between 0.3 and 0.5 in postmenopausal women, tended to be low in Lab C (ranging from 0.2–0.3), but varied from 0.2 to 1.5 in Lab A and 0.3 to 1.3 in Lab B. Levels of androstenedione were relatively consistent between labs among menstruating women but varied 2-fold between labs for postmenopausal women. Labs A and B reported a similar percentage of free E_2 values, but Lab C determinations were consistently higher. Values of albumin-bound E_2 varied considerably between labs and between menstrual groups except for Lab B, which reported relatively similar values in all premenopausal women. SHBG levels were similar for Labs B and C.

In general, the SDs for estimates of serum estrone and estradiol tended to rise in proportion to the mean values in all the labs, suggesting that, for these hormones, the variance is dependent on the concentration of hormone. Estrone data are plotted in Fig. 1 according to laboratory using both the raw values (1a) and the natural logarithms (1b). The log transform removes the dependence on concentration, normalizes the data, and permits the use of parametric models for analysis. Consequently, results for estrone and estradiol will be presented both with and without log transformation.

If we ignore the between-subject variability, then the overall CVs can be computed. Table 3 shows widely varying overall CVs between laboratories with many values greater than 15%. The CVs for estrone and estradiol were 20% or more in two labs and similarly high for albumin-bound E_2 in all labs. Lab C consistently had lower CVs than the other two laboratories, with the exception of SHBG.

The intraassay and interassay CVs are shown in Table 4. With the exception of SHBG, hormone values were generally similar within and between days for Lab C, and CVs were 10% or lower for most hormones. Measurements of estrone and estradiol from Labs A and B were more variable than those from Lab C, although when analyzed on a log scale, the CVs for these estrogens were low in all labs. For some hormones, the interassay CVs were twice the magnitude of the intraassay CVs. Consistently low CVs were observed for the percentage of free E_2 levels for all labs. Intraassay and interassay CVs among postmenopausal women were higher than those for premenopausal women and varied considerably between labs, whereas CVs for midfollicular and preovulatory women tended to be similar to the CVs reported in Table 4 (results not shown).

No lab assayed any hormone in all menstrual groups consistently from one day to the next (Table 5), although significant day-to-day variability appeared less problematic among mid-follicular phase women. In this group, hormones measured consistently over time included androstenedione in all labs, estrone from Labs A and C, and estradiol in Labs B and C. Significant day-to-day variability was observed among preovulatory phase women for estrone levels in all labs and for estradiol and androstenedione in Labs A and B. Results for postmenopausal women also differed over time, with for instance, significant variability for estrone and SHBG in all labs, and estradiol, androstenedione, and albumin-bound E_2 in Labs A and C. Adjustment for variable cholesterol and triglyceride concentrations did not materially alter these findings.

DISCUSSION

Few studies to date have reported on the error associated with laboratory measurements of serum hormone levels in conjunction with the conduct of large scale epidemiological studies. Without consistent laboratory results, individuals may be incorrectly ranked in terms of their hormone levels, and the ability to detect an association with disease will be hampered. When laboratory results are reproducible but do not reflect the true underlying hormone values, results cannot be compared from studies using different laboratories, and the interpretation of any result becomes questionable. Our results reveal substantial differences between laboratories in the absolute values of the hormones studied, particularly for estrone and estradiol. Moreover, for each laboratory, day-to-day variability was considerable.

Prior to conducting this pilot study, the three laboratories reported

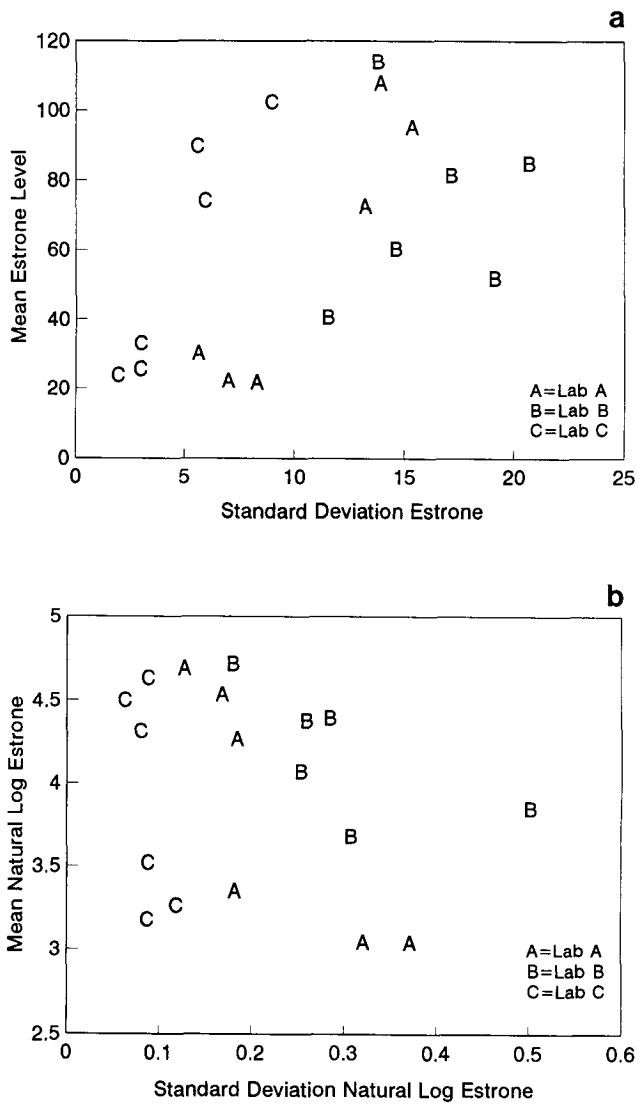

Fig. 1. *a*, mean by SD of estrone; *b*, mean by SD of the natural log of estrone.

Table 3 *Overall CVs by laboratory (Hormone Feasibility Trial)*

Hormone	Lab A	Lab B	Lab C
Estrone	20	25	9
Estradiol	29	24	12
Log estrone	7	8	2
Log estradiol	9	7	3
Androstenedione	24	9	–[a]
% Free E_2	8	12	3
Albumin-bound E_2	39	25	16
SHBG	19	14	23

[a] Overall CV could not be calculated because the ANOVA model produced a negative estimate for day-to-day variance.

Table 4 *Intraassay and interassay CVs by laboratory (Hormone Feasibility Trial)*

Hormone	Lab A	Lab B	Lab C
Estrone			
Intraassay	12	13	7
Interassay	16	21	7
Estradiol			
Intraassay	15	14	9
Interassay	24	19	8
Log estrone			
Intraassay	3	4	1
Interassay	6	7	2
Log estradiol			
Intraassay	4	5	2
Interassay	8	5	3
Androstenedione			
Intraassay	16	7	9
Interassay	18	6	–[a]
% Free E_2			
Intraassay	6	8	3
Interassay	5	8	2
Albumin-bound E_2			
Intraassay	15	13	14
Interassay	36	21	9
SHBG			
Intraassay	13	6	9
Interassay	14	12	21

[a] Interassay CV could not be calculated because the ANOVA model produced a negative estimate for day-to-day variance.

intraassay CVs of 11% or lower and often less than 5%, with interassay CVs generally less than 15%. Our intraassay CVs tended to be higher than those reported by the laboratories, particularly for estrone and estradiol in Labs A and B and albumin-bound E_2 in all labs. The interassay CVs were generally similar to reported values, which ranged from 4 to 15%, but values greater than 20% were observed. Based on our calculations, interassay CVs for Lab C were lower than their reported values for most hormones. The combination of low postmenopausal estradiol values and low CVs suggested that estradiol values from Lab C may be more accurate than the estimates from the other labs.

With few exceptions, the overall CVs were greater than 3%, which is the recommended value for cholesterol laboratories (14). This may be expected since the concentration of sex hormones are considerably lower (pg) than cholesterol (mg), requiring assays with greater complexity. However, the comparison provides a useful illustration of the impact of assay variability. The cholesterol guidelines for clinical application were made taking into account biological and laboratory variability, were based on population data, and only used laboratories with low CVs (14, 15). Regardless of the differences in assay complexity and sensitivity between cholesterol and sex steroids, the implication for epidemiological studies is the same. If small differences in hormone levels are important in cancer etiology, similar to the role of small differences in cholesterol levels on cardiovascular disease, then present steroid hormone methodologies would hamper the observation of such relationships. Indeed, some have speculated that steroid concentration differences of 11 to 20% may be associated with substantial differences in breast cancer risk (16–18). Although the biological plausibility of cancer risk related to such small differences in hormone levels remains controversial, present laboratory methods make testing of such hypotheses difficult.

Hormones from mid-follicular phase women were most likely to be measured consistently from day-to-day compared to hormones from women in the other two menstrual groups. This may be explained in part because mid-follicular phase estrogens, particularly estradiol, are in one of the more stable parts of the laboratories' standard curve and well above the limit of sensitivity. In contrast, values for postmenopausal women were not similar form day-to-day, presumably because these measurements are near the limit of sensitivity, making random errors more likely. Significant day-to-day variability can be problematic to epidemiological studies, where large numbers of samples are analyzed over several months or years and where differences between persons are small, such as estradiol in postmenopausal women. Grouping cases and controls, or ideally, analyzing matched case-control samples on the same day can minimize this problem.

In this study, the absolute values for the hormones varied substantially between laboratories, which implies that some or all must be different from the true values. Furthermore, there was disparity in the relative difference in values between women in the same menstrual phase. For postmenopausal women, the relative difference in estrone values within laboratories varied from 0% in one lab to nearly 25% in

Table 5 *Significance of interassay hormone variability by menstrual group (Hormone Feasibility Trial)*

Hormone	Lab A	Lab B	Lab C
Estrone			
Follicular	ns	*a*	ns
Preovulatory	*a*	*b*	*a*
Postmenopausal	*b*	*a*	*b*
Estradiol			
Follicular	*b*	ns	ns
Preovulatory	*b*	*b*	ns
Postmenopausal	*a*	ns	*a*
Log Estrone			
Follicular	ns	*a*	*a*
Preovulatory	*a*	*b*	*a*
Postmenopausal	*b*	*a*	*a*
Log Estradiol			
Follicular	*b*	ns	ns
Preovulatory	*b*	*b*	ns
Postmenopausal	*b*	ns	*a*
Androstenedione			
Follicular	ns	ns	ns
Preovulatory	*b*	*a*	ns
Postmenopausal	*a*	ns	*a*
% Free E_2			
Follicular	ns	*a*	*b*
Preovulatory	ns	ns	*a*
Postmenopausal	*a*	*a*	ns
Albumin-bound E_2			
Follicular	*b*	ns	*a*
Preovulatory	*b*	*a*	ns
Postmenopausal	*a*	ns	*a*
SHBG			
Follicular	ns	*b*	*a*
Preovulatory	ns	*b*	*b*
Postmenopausal	*a*	*b*	*b*

[a] $P < 0.05$; ns, not significant.
[b] $P < 0.005$.

another. Thus, for estrone, the ranking of women would depend on which laboratory analyzed the samples. Although evaluation of means suggested an adequate general ranking of these six individuals, the raw data of single estimates would not rank them consistently. In epidemiological investigations relative risks are based on comparisons between groups with low and high hormone levels. For these risk estimates to be valid, it is critical that subjects are ranked correctly. Our findings suggest that efforts to summarize the literature, to conduct meta-analyses, or to compare results across populations when different laboratories have been used for hormone assays could be invalid. Although standardization of laboratories is a complex and difficult issue, a useful initial step would be to evaluate the validity of hormone results across laboratories.

It was not the intention of this study to address specific laboratory procedures associated with error. A few of the possible differences between laboratories include differences in computer reduction of the raw data, differences in the analyte detected by each antibody, differences in the usable range of each standard curve and differences in extraction efficiences. One previous report (19) found markedly varied estradiol values using a standard radioimmunoassay kit and suggested that some unidentified compound(s) may cross-react with the antibody. In another study (8), investigators checked cross-reactivity by multiple assays of chromatographic peaks to eliminate errors due to failure of the column separation. Further investigations into the problems and possible solutions for laboratory testing of steroid hormones should improve the accuracy, and thus the utility, of these measures.

In general, previous clinical and international studies (5, 6, 20–22) have not transformed hormone data. Our data, as well as some recent epidemiological analyses (7, 10, 16, 23), suggest that a log transformation should be applied to steroid hormone data, since it removes the dependence of the variance on the mean, normalizes the data, and reduces the CVs. It is unclear, however, what impact this would have on comparisons across groups.

Our study evaluated a limited number of hormones and related compounds among a small number of women and found that current laboratory error is not negligible and may diminish the ability to observe disease-hormone associations in epidemiological studies, particularly those conducted on postmenopausal women. Moreover, the values of these hormones differed substantially between laboratories, highlighting the need for standardization of these hormone assays. Future collaboration between endocrine scientists and epidemiologists would strengthen epidemiological research by improving the precision and accuracy of measurements for these steroid hormones and binding proteins.

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